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Characterization of the V_0 Domain of the Coated Vesicle (H^+)-ATPase*

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Jimin Zhang, Melanie Myers‡, and Michael Forgac§

From the Department of Cellular and Molecular Physiology, Tufts University School of Medicine, Boston, Massachusetts 02111

The coated vesicle (H^+)-ATPase is composed of two domains, a peripheral V_1 domain containing the 73 (A subunit)-, 58 (B subunit)-, 40-, 34-, and 33-kDa subunits and an integral V_0 domain containing the 100-, 38-, 19-, and 17 (c subunit)-kDa subunits (Adachi, I., Puopolo, K., Marquez-Sterling, N., Arai, H., and Forgac, M. (1990) *J. Biol. Chem.* 265, 967–973). In the present manuscript we characterize the V_0 domain with respect to its structural and activity properties. Glycerol density gradient separation of solublized coated vesicle membrane proteins reveals the presence of an excess of V_0 domains which migrate with a molecular weight of 250,000 and contain the V_0 polypeptides in the same stoichiometry as in the intact V_1V_0 complex. Like the c subunit in V_1V_0, the c subunit of the free V_0 domain is labeled by [^{14}C]*N,N'*-dicyclohexylcarbodiimide (DCCD) and is extracted by chloroform:methanol. In addition, a monoclonal antibody specific for the 100-kDa subunit of the intact (H^+)-ATPase recognizes the 100-kDa subunit of V_0. Tryptic cleavage of the V_0 complex gives the same pattern of fragments for the 100- and 38-kDa subunits as in the intact complex, but with an increase in sensitivity, suggesting greater exposure of these subunits in free V_0. Proton conduction was measured in reconstituted vesicles containing the V_0 domain and in native vesicles stripped of V_1. No DCCD-inhibitable proton conduction was observed in either preparation, suggesting that unlike the corresponding F_0 domain of F_1F_0, the free V_0 domain is not an open proton channel.

The vacuolar (H^+)-ATPases[1] are responsible for acidification of intracellular compartments in eukaryotic cells, a function that is crucial for a variety of cellular processes (1–3). Vacuolar (H^+)-ATPases have been isolated from a number of sources, including clathrin-coated vesicles (4), chromaffin granules (5, 6), kidney microsomes (7), and the vacuoles of *Neurospora* (8), plants (9, 10), and yeast (11, 12).

In addition to sharing many structural and functional properties (1), the vacuolar (H^+)-ATPases also closely resemble the F_1F_0 class of (H^+)-ATPases (13–16). This is true in terms of both the overall structure (17–19) and in the sequence homology observed between certain subunits (20–26). Thus the A and B subunits of the V-ATPases and the α and β subunits of F_1 are all derived from a common ancestral nucleotide binding protein (20–25), whereas the c subunit of the V-ATPases appears to have arisen by a gene duplication and fusion of the gene encoding the corresponding c subunit of F_0 (26).

Like the F-type ATPases, the V-ATPases are composed of a peripheral set of polypeptides (termed V_1) and an integral set of polypeptides (termed V_0) (19). We have demonstrated previously that these two domains could be dissociated and reassembled into a functional (H^+)-ATPase (27). Because of the similarity between the V- and F-type ATPases, it was of interest to determine whether, as with the F_0 domain, the V_0 domain remained assembled as a discrete macromolecular complex in detergent. We also wished to compare the structural properties of the V_0 subunits in the free V_0 domain with those observed in the intact V_1V_0 complex. Finally, by analogy with F_0, we have investigated the ability of the V_0 domain to act as a passive DCCD-inhibitable proton channel.

EXPERIMENTAL PROCEDURES

Materials—Calf brains were obtained fresh from a local slaughterhouse. $C_{12}E_9$, cholesterol, cholic acid, potassium iodide, DCCD, and ATP (grade II) were purchased from Sigma. Phosphatidylcholine and phosphatidylserine were obtained as chloroform solutions from Avanti Polar Lipids, Inc. and stored at −20 °C. Acridine orange was purchased from Eastman Kodak and 9-amino-6-chloro-2-methoxyacridine (ACMA) was purchased from Molecular Probes. *p*-Nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt), and affinity-purified goat anti-mouse IgG conjugated to alkaline phosphatase were obtained from Bio-Rad.

Preparation of Stripped Vesicles—Clathrin-coated vesicles were prepared from calf brain as described previously (28). Vesicles were stripped of their clathrin coat by dilution (40-fold) into 5 mM Tris (pH 8.5), 150 mM sucrose, and 0.5 mM EDTA followed by incubation for 1 h at 23 °C and sedimentation for 1 h at 100,000 × *g*. Stripped vesicles displayed a 10-fold higher specific activity for the (H^+)-ATPase relative to intact coated vesicles.

Glycerol Density Gradient Separation of V_1V_0 and V_0 Complexes—Glycerol density gradient sedimentation was carried out by a modification of the procedure described previously (4). Stripped vesicles (1.0 mg of protein/ml) were solubilized with 1.0% $C_{12}E_9$ containing 0.4 mg of phosphatidylcholine/ml and 0.2 mg of phosphatidylserine/ml in solubilization buffer (50 mM NaCl, 30 mM KCl, 20 mM HEPES (pH 7.0), 0.2 mM EGTA, 10% glycerol, 2 mM 2-mercaptoethanol) for 5 min at 23 °C followed by 15 min at 4 °C. The insoluble material was removed by sedimentation at 150,000 × *g* for 1 h in a Beckman SW 50.1 rotor, and 0.5 ml of the solubilized mixture was applied to an 11-ml 12–25% linear glycerol gradient prepared in solubilization buffer containing 0.02% $C_{12}E_9$, 8 μg of phosphatidylcholine/ml and spun for 16 h at 38,000 rpm in a Beckman SW-41 rotor. The resultant gradients were fractionated from the bottom using a peristaltic pump to give 19–20 fractions of 0.55–0.60 ml/fraction. The intact V_1V_0 complex, which has a molecular weight 700,000–750,000 (17), typically peaks in fractions 4–6, whereas the dissociated V_0 domain, containing

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‡ Medical Foundation Postdoctoral Fellow.

§ American Heart Association Established Investigator. To whom correspondence should be addressed.

[1] The abbreviations used are: (H^+)-ATPase, proton-translocating adenosine triphosphatase; $C_{12}E_9$, polyoxyethylene-9-lauryl ether; ACMA, 9-amino-6-chloro-2-methoxyacridine; DCCD, *N,N'*-dicyclohexylcarbodiimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; CCCP, carbonyl cyanide *p*-chlorophenylhydrazone.

the 100-, 38-, 19-, and 17-kDa subunits, typically peaks in fractions 10–12. Comparison with the sedimentation behavior of proteins of known molecular weight indicates the free V_0 complex has a molecular weight of approximately 250,000 (data not shown).

Dissociation of the (H^+)-ATPase V_1 Domain—Dissociation of the peripheral V_1 domain was carried out essentially as described previously (27). Stripped vesicles (1.0 mg of protein/ml) were treated with 0.40 M KI and 5.0 mM ATP in buffer containing 20 mM HEPES (pH 7.0), 0.2 mM EGTA, and 2 mM 2-mercaptoethanol for 1 h at 4 °C and then sedimented for 1 h at 150,000 × *g* in a Beckman SW-50.1 rotor. The KI/ATP stripped membranes were then solubilized with $C_{12}E_9$ and the solubilized proteins separated by glycerol density gradient sedimentation as described above.

[^{14}C]DCCD Labeling of the 17-kDa c Subunit of the V_0 Domain—[^{14}C]DCCD labeling of the 17-kDa c subunit of the V_0 domain was carried out as described previously for labeling of the c subunit of the intact V_1V_0 complex (29). To 500 μl of the peak V_0 containing fraction from the glycerol density gradient (6.0 μg of protein) was added 50 μM [^{14}C]DCCD (specific activity 55 mCi/mmol), and the reaction was allowed to proceed for 30 min at 23 °C. The reaction was then stopped by precipitation of the protein using 6% trichloroacetic acid, incubation for 30 min at 4 °C, sedimentation for 5 min at 10,000 × *g*, solubilization of the precipitated protein using Laemmli sample buffer and separation of the solubilized polypeptides on a 13.5% acrylamide gel as described below. Following electrophoresis, the gel was washed for 15 min in 10% acetic acid, 10% isopropyl alcohol, impregnated with ENHANCE (Du Pont-New England Nuclear), dried under vacuum, and exposed to Kodak XAR-5 film for 7 days at −70 °C using an intensifier screen.

Chloroform:Methanol Extraction of the 17-kDa c Subunit of V_0—Chloroform:methanol extraction was carried out essentially as described previously (29). 400 μl of the V_0 containing fraction (4.8 μg of protein) was placed in a 15-ml conical glass centrifuge tube to which was added 5 volumes of chloroform:methanol (2:1, v/v). The sample was vortexed vigorously and incubated on ice, with occasional vortexing, for 1 h. The sample was then spun for 5 min at 5,000 × *g* in a table top centrifuge, the upper aqueous phase and the interface (containing most of the denatured protein) was carefully removed, and the lower organic phase was transferred to a separate tube and dried under nitrogen. The extracted protein was then solubilized using 200 μl of Laemmli sample buffer and 30 or 60 μl were run on a 12.5% acrylamide gel as described below.

Western Blot Analysis—The presence of a 100-kDa polypeptide in the V_0 domain capable of reacting with a monoclonal antibody specific for the 100-kDa subunit of the intact V_1V_0 complex was demonstrated by Western blot analysis as follows. Membranes from which the clathrin had been stripped (1 mg of protein/ml) were solubilized with $C_{12}E_9$ and the solubilized proteins separated by glycerol density gradient sedimentation as described above. 60-μl aliquots of each fraction were solubilized with Laemmli sample buffer and applied to a 12% acrylamide gel. After electrophoresis, the proteins were transferred electrophoretically to nitrocellulose by blotting at 100 mA for 16 h at 4 °C using a Bio-Rad Trans Blot cell and a transfer buffer containing 25 mM Tris, 192 mM glycine (pH 8.3), 20% methanol.

After transfer, the blot was washed twice in TBS (20 mM Tris (pH 7.5), 0.5 M NaCl) and then incubated for 30 min in TBS containing 1.5% gelatin. After blocking, the blot was washed twice in TTBS (TBS containing 0.05% Tween 20) followed by incubation for 2 h in TTBS containing 0.5% gelatin and a 1:50 dilution of the monoclonal antibody 3A-6D. The blot was then washed three times in TTBS and incubated for 1 h in TTBS containing 0.5% gelatin and a 1:1000 dilution of a goat anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad). After incubation with the secondary antibody, the blot was washed once in TTBS and twice in TBS and then developed by incubation for 20 min in 0.1 M sodium carbonate (pH 9.8), 1 mM $MgCl_2$ containing 0.03% *p*-nitro blue tetrazolium phosphate (toluidine salt) and 0.015% 5-bromo-4-chloro-3-indolyl phosphate. The blot was then washed with water and air-dried.

Proteolysis of the V_0 Domain—The gradient fractions containing either the intact V_1V_0 complex (1.8 μg of protein) or the V_0 domain (1.2 μg of protein) were treated with 0–1.0 μg of trypsin for 4 h at 23 °C. The proteolysis was then stopped by addition of 5 mM TLCK followed by SDS-PAGE on a 12% acrylamide gel and silver staining as described below.

Reconstitution of the V_0 Domain into Phospholipid Vesicles—The procedure used in reconstitution of the V_0 domain is based on the protocol employed to reconstitute the intact V_1V_0 complex (4). The glycerol density gradient fractions containing the peak of the V_0 polypeptides (typically fractions 10–12) from either native membranes or membranes treated with KI/ATP were concentrated 2-fold using a Centricon 10 microconcentrator. To 1.0 ml of the concentrated V_0 was added 0.2 ml of 5% cholate, 4 mg of phosphatidylcholine/ml, and 2 mg of phosphatidylserine/ml in solubilization buffer followed by 0.34 ml 10% cholate, 4 mg of cholesterol/ml and 7 mg of phosphatidylcholine/ml in 150 mM NaCl, 2 mM 2-mercaptoethanol. The mixture was incubated for 5 min at 23 °C and then 10 min at 4 °C followed by dialysis for 2 days against five changes of 200 volumes of solubilization buffer using Spectrapor-2 dialysis tubing with a M_r cutoff of 12,000–14,000. As previously described, reconstitution of the intact V_1V_0 complex under these conditions gave rise to reconstituted vesicles capable of ATP-dependent proton uptake as assayed by uptake of acridine orange (4).

Measurement of Passive Proton Flux in Reconstituted Vesicles Containing the V_0 Domain or Native Membranes from Which the V_1 Domain Had Been Dissociated—The V_0 domain (12 μg of protein) was reconstituted into phospholipid vesicles as described above except the dialysis buffer contained 150 mM KCl, 20 mM HEPES (pH 7.0), 0.2 mM EGTA, 2 mM 2-mercaptoethanol, 10% glycerol. The vesicles were then diluted 1:20 into 150 mM NaCl, 20 mM HEPES (pH 7.0), 0.2 mM EGTA, 2 mM 2-mercaptoethanol, 10% glycerol, 0.5 mg of bovine serum albumin/ml, and 2 μM ACMA. To initiate proton flux, a membrane potential was generated by addition of 20 nM valinomycin. The resultant proton uptake was monitored by fluorescence quenching of ACMA using excitation and emission wavelengths of 410 and 490 nm, respectively. As a negative control, reconstituted vesicles lacking protein were prepared and assayed in an identical manner. As a positive control, each vesicle preparation was tested for its ability to generate a membrane potential driven proton flux following addition of 1.0 μM of the proton ionophore CCCP.

Stripped vesicles (1.5 mg of protein/ml) that had been treated with KI and ATP as described above were loaded with potassium by permeabilization with 0.7% cholate, 0.4 μg of phosphatidylcholine/ml, 0.2 μg of phosphatidylserine/ml followed by dialysis against four changes of 200 volumes of 100 mM K_2SO_4, 20 mM HEPES (pH 7.0), 0.2 mM EGTA, 10% glycerol, 2 mM 2-mercaptoethanol over 2 nights. Potassium sulfate rather than potassium chloride was employed in experiments with native vesicles to avoid dissipation of the K^+/valinomycin-induced membrane potential by the chloride channel which is present in these vesicles (30, 31). Proton flux was then measured by ACMA quenching in response to a potassium/valinomycin-induced membrane potential as described above. As a negative control, a second batch of vesicles was prepared in an identical fashion except that they were treated with 50 μM DCCD for 1 h at 4 °C prior to measurement of proton flux. Membrane potential driven proton flux on addition of CCCP was also tested as described above.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out using 12, 12.5, or 13.5% acrylamide gels by the method of Laemmli (32). Following electrophoresis, gels were fixed for at least 2 h in 30% methanol, 7.5% acetic acid prior to silver staining by the method of Oakley *et al.* (33).

RESULTS

Fig. 1*A* shows the protein pattern obtained after solubilization of coated vesicle membrane proteins with $C_{12}E_9$, separation on 12–25% glycerol density gradients, and SDS-PAGE. As can be seen, the V_0 subunits of molecular masses 100, 38, 19, and 17 kDa migrate at two distinct positions on the density gradients. The heavier complex, which sediments with a molecular weight of 700,000–750,000 (17), corresponds to the intact V_1V_0 complex and thus has the complete complement of nine subunits. The lighter complex (fractions 9–11), which by comparison with the V_1V_0 complex and other marker proteins, has a molecular weight of approximately 250,000, contains the V_0 subunits but not the V_1 subunits. There are also present a variety of contaminating proteins which do not peak with the V_0 subunits. We have demonstrated previously that the V_0 subunits are present in the V_1V_0 complex with a stoichiometry of $(100{,}000)_1$, $(38{,}000)_1$, $(19{,}000)_1$, and $(17{,}000)_6$ (17). As can be seen by comparing the relative staining intensities of the V_0 subunits in the two complexes, this same stoichiometry appears to apply to the free V_0 domain. If the vesicles are first treated with KI and ATP (Fig. 1*B*), the V_0

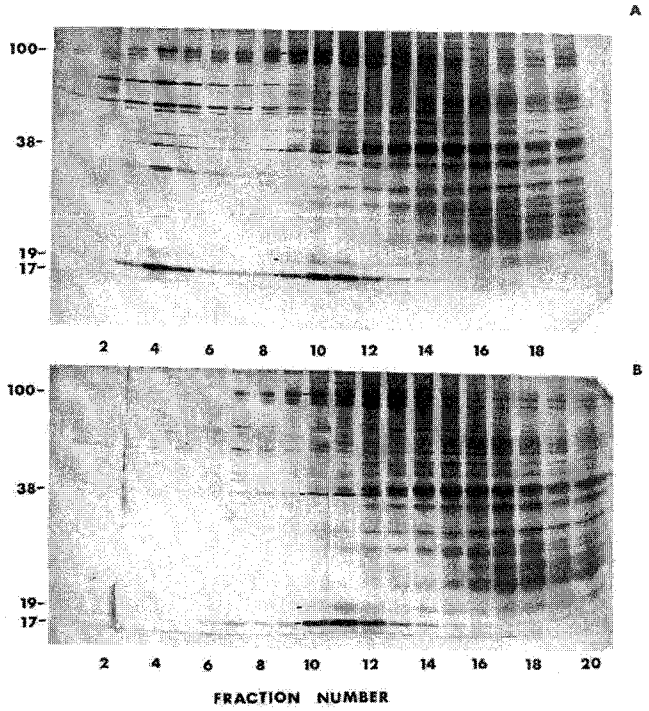

FIG. 1. **Glycerol density gradient separation of $C_{12}E_9$ solubilized V_1V_0 and V_0 complexes.** *A*, stripped vesicles (1.0 mg of protein/ml) were solubilized with $C_{12}E_9$ and the solubilized proteins separated by sedimentation on a 12–25% glycerol density gradient as described under "Experimental Procedures." 60 μl of each fraction was applied to a 12% acrylamide gel, and SDS-PAGE was carried out by the method of Laemmli (32). The bottom of the gradient corresponds to fraction 1, and the molecular masses of the V_0 subunits (in kDa) are indicated to the *left*). The V_0 subunits peak in fraction 4, corresponding to the intact V_1V_0 complex, and fraction 10, corresponding to the free V_0 complex. *B*, stripped vesicles (1.0 mg of protein/ml) were treated with KI and ATP as described under "Experimental Procedures" and then solubilized with $C_{12}E_9$ and separated by glycerol density gradient sedimentation as described in *A*. The V_0 subunits appear only in the lighter complex which, on this gradient, peaks in fraction 11.

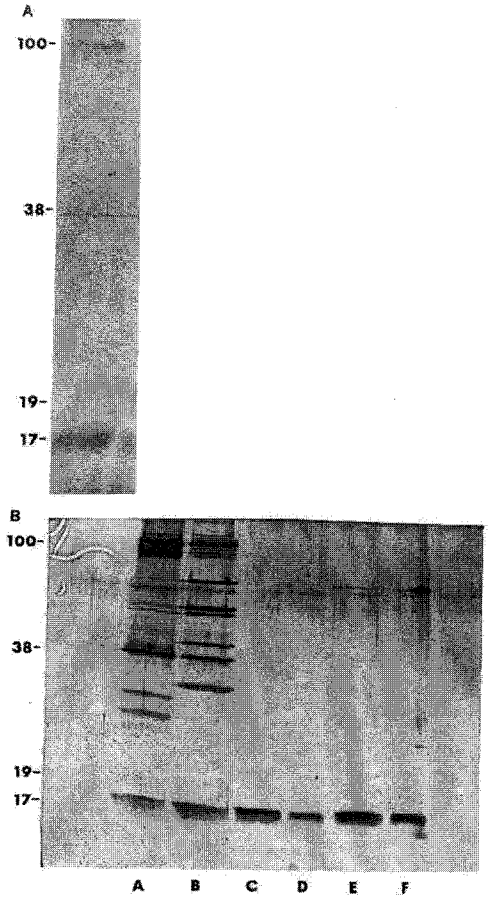

FIG. 2. **[^{14}C]DCCD labeling and chloroform:methanol extraction of the 17-kDa c subunit of the V_0 complex.** *A*, 500 μl of the peak V_0 fraction (6.0 μg protein) was reacted with 50 μM [^{14}C] DCCD for 30 min at 23 °C followed by precipitation with trichloroacetic acid, separation on a 13.5% acrylamide gel, and autoradiography as described under "Experimental Procedures." *B*, 400 μl of the peak V_0 fraction (4.8 μg of protein) or the peak V_1V_0 fraction (7.2 μg of protein) were extracted with 5 volumes of chloroform:methanol (2:1, v/v) and the proteins extracted into the organic phase solubilized in 200 μl of Laemmli sample buffer as described under "Experimental Procedures." *Lanes C* and *D*, 60 and 30 μl of the protein extracted from the V_0 fraction, respectively; *lanes E* and *F*, 60 and 30 μl of the protein extracted from the V_1V_0 fraction, respectively. *Lane A*, an aliquot of the unextracted V_0 fraction equivalent to that employed in *lane D*. *Lane B*, an aliquot of the unextracted V_1V_0 fraction equivalent to that employed in *lane F*. The molecular masses of the V_0 subunits (in kDa) are shown to the *left*.

subunits migrate entirely in the lighter complex. The slight shift in position of the V_0 complex in Fig. 1*B* relative to Fig. 1*A* corresponds to small variations between individual glycerol density gradient runs. These results suggest that the lighter complex corresponds to the free V_0 domain which remains assembled after either detergent solubilization or KI/ATP treatment and that there is an excess of V_0 domains in the native vesicle over that required to form functional V_1V_0 complexes.

To test whether the 100-, 38-, 19-, and 17-kDa polypeptides migrating as a complex of 250,000 correspond to the authentic V_0 subunits, the following tests were performed. First, the ability of [^{14}C]DCCD to label the 17-kDa (c) subunit was tested. As can be seen in Fig. 2*A*, the c subunit of V_0, like that in the intact V_1V_0 complex (29), was labeled by [^{14}C]DCCD. In addition to labeling of the 17-kDa c subunit, a small amount of label was also observed at approximately 100 kDa. This may correspond to some labeling of the 100-kDa subunit, labeling of a different 100-kDa polypeptide, or to partial aggregation of the 17-kDa subunit, which is present in a stoichiometry of six copies per complex (17). In addition to labeling by [^{14}C]DCCD, the 17-kDa polypeptide present in the V_0 containing fraction was extracted by chloroform:methanol (Fig. 2*B*), as demonstrated previously for the authentic c subunit (29). Thus in both its reactivity toward DCCD and its hydrophobicity, the c subunit of the V_0 complex was indistinguishable from the c subunit of V_1V_0.

As a further test of the relationship between these two complexes, Western blot analysis was performed using a monoclonal antibody specific for the 100-kDa subunit of the coated vesicle (H^+)-ATPase. Fig. 3 shows that this monoclonal antibody recognized the 100-kDa subunit in both the intact V_1V_0 complex and in isolated V_0. Silver staining of a parallel SDS-PAGE gel run on the same gradient fractions indicated that, for this glycerol density gradient, the 38-, 19-, and 17-kDa polypeptides, like the 100-kDa immunoreactive band, peaked in fractions 6 and 12 (data not shown). This data provide strong evidence for the identity of the 100-kDa polypeptide in these two complexes.

To further characterize the V_0 domain, proteolysis was carried out on both the V_1V_0 and V_0 complexes in the detergent solubilized state. We have demonstrated previously that

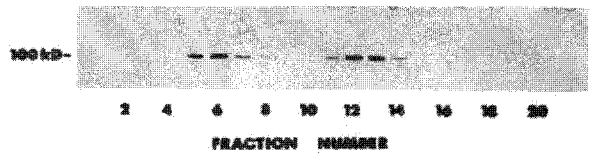

FIG. 3. **Western blot analysis of glycerol density gradient fractions of $C_{12}E_9$-solubilized stripped vesicle membrane proteins using the 100-kDa-reactive monoclonal antibody 3A-6D.** Stripped vesicles (1.0 mg of protein/ml) were solubilized with $C_{12}E_9$ and the solubilized proteins separated by glycerol density gradient sedimentation as described in Fig. 1*A*. 60 μl of each fraction was applied to a 12% acrylamide gel and SDS-PAGE, and electrophoretic transfer to nitrocellulose and Western blotting using the monoclonal antibody 3A-6D and goat anti-mouse IgG conjugated to alkaline phosphatase were carried out as described under "Experimental Procedures." Silver staining of a second polyacrylamide gel run on the same gradient fractions indicated that the 38-, 19-, and 17-kDa polypeptides, like the 100-kDa immunoreactive band, peaked in fractions 6 and 12.

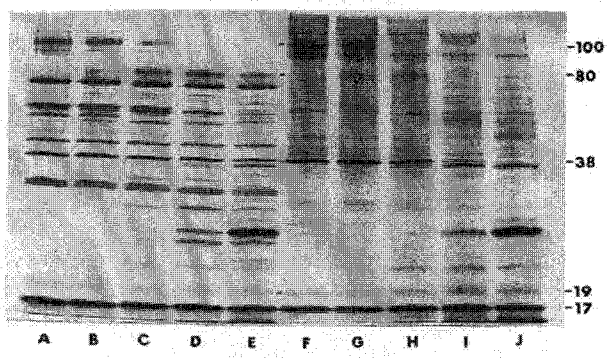

FIG. 4. **Trypsin digestion of detergent-solubilized V_1V_0 and V_0 complexes.** Peak gradient fractions containing either intact V_1V_0 complex (*lanes A–E*) (1.8 μg of protein) or the free V_0 domain (*lanes F–J*) (1.2 μg of protein) were treated with 0 (*lanes A* and *F*), 0.001 (*lanes B* and *G*), 0.01 (*lanes C* and *H*), 0.10 (*lanes D* and *I*), or 1.0 (*lanes E* and *J*) μg trypsin for 4 h at 23 °C. Proteolysis was stopped by addition of 5 mM TLCK, and the samples were run on a 12% acrylamide gel followed by silver staining as described under "Experimental Procedures." The positions of the V_0 subunits and the 80-kDa fragment of the 100-kDa subunit are indicated at the *right* of the figure.

trypsin treatment of the intact (H^+)-ATPase generates an 80-kDa fragment of the 100-kDa subunit and cleaves the 38-kDa polypeptide approximately 1–2 kDa from the amino terminus (18). Moreover, both of these cleavage sites were shown to be present on the cytoplasmic side of the membrane (18). As can be seen in Fig. 4, the same proteolytic cleavage pattern is obtained for the 100- and 38-kDa polypeptides in the free V_0 domain. The fact that these cuts happen at somewhat lower trypsin concentrations for V_0 than for V_1V_0 suggests that there is greater accessibility of the protease to these polypeptides in the free V_0 complex. That the same pattern is observed for both detergent-solubilized and reconstituted V_0 (data not shown) indicates that, like the intact V_1V_0 complex, the free V_0 domain reconstitutes primarily with the cytoplasmic face exposed.

To test whether the V_0 domain could function as a passive proton channel, the following protocol was employed. The detergent-solubilized V_0 domain from either untreated vesicles or vesicles which had been treated with KI and ATP was isolated by density gradient sedimentation as shown in Fig. 1 and then reconstituted into phospholipid vesicles by cholate dialysis as described previously (4). The vesicles were reconstituted in the presence of 150 mM KCl and then diluted into a potassium free buffer in order to establish a large potassium gradient across the membrane. The vesicles were incubated in the presence of the fluorecence dye ACMA, and the assay was initiated by addition of valinomycin. Because of the substantial potassium gradient present across the vesicle membrane, addition of valinomycin generated a negative interior membrane potential which acted as a driving force for proton uptake, which was monitored by fluorescence quenching of ACMA. Vesicles lacking protein acted as the negative control, whereas addition of the proton ionophore CCCP acted as the positive control. As can be seen in Fig. 5*A*, no greater passive proton transport was observed in vesicles containing V_0 than was observed in vesicles lacking protein, despite the substantial fluorescence quenching observed on addition of CCCP. Moreover, treatment of reconstituted V_0 with DCCD had no effect on this low background level of proton leakage (data not shown).

Because it is possible that the V_0 domain may have become inactivated during the course of isolation and reconstitution, we also tested to see whether the V_0 domain remaining in the native membrane after dissociation of V_1 with KI and ATP could conduct protons. Native vesicles were loaded with potassium by permeabilization with cholate plus phospholipid followed by removal of the detergent by dialysis. We have observed that cholate is ineffective at solubilization of either the V_0 domain or the intact V_1V_0 under these conditions (data not shown). As shown in Fig. 5*B*, the V_0 domain in the native membrane is also incapable of DCCD-inhibitable proton translocation, despite the fact that, as we have demonstrated previously (27), this V_0 is competent to reassemble with the V_1 domain to give an active (H^+)-ATPase complex.

DISCUSSION

The V_0 domain of the coated vesicle (H^+)-ATPase is composed of four subunits of molecular weight 100,000, 38,000, 19,000, and 17,000 (19). They are operationally defined as integral subunits by the observation that they remain attached to the membrane after dissociation of the peripheral V_1 subunits by chaotropic agents such as KI and KNO_3 (19). In the present manuscript we provide the first demonstration that these integral subunits remain assembled as a complex following detergent solubilization. This complex has an approximate molecular weight of 250,000 and, by comparison with the intact V_1V_0 complex (17), has a subunit stoichiometry of one copy each of the 100-, 38-, and 19-kDa subunits and six copies of the 17-kDa subunit. The ability of the V_0 subunits to remain assembled as a complex in detergent solution has also recently been reported for the vacuolar (H^+)-ATPase from *Neurospora* (34).

As can be seen from Fig. 1, coated vesicles appear to contain an excess of V_0 domains over what is required to form functional V_1V_0 complexes. It was therefore important to compare the properties of the polypeptides present in these "excess" V_0 domains with the "authentic" V_0 subunits to determine whether they were the same. According to the following criteria, the V_0 polypeptides present in these two populations of V_0 are identical: 1) [^{14}C]DCCD labeling and chloroform:methanol extraction of the 17-kDa c subunit; 2) reaction of the 100-kDa polypeptide with a specific monoclonal antibody by Western blot; 3) tryptic cleavage pattern of both the 100- and 38-kDa subunits. With respect to proteolysis, it is interesting to note that although the same tryptic fragments of the 100- and 38-kDa subunits are generated, proteolysis appears to occur at lower trypsin concentrations for the free V_0 domain relative to the intact V_1V_0 complex, suggesting that removal of the V_1 domain has increased the accessibility

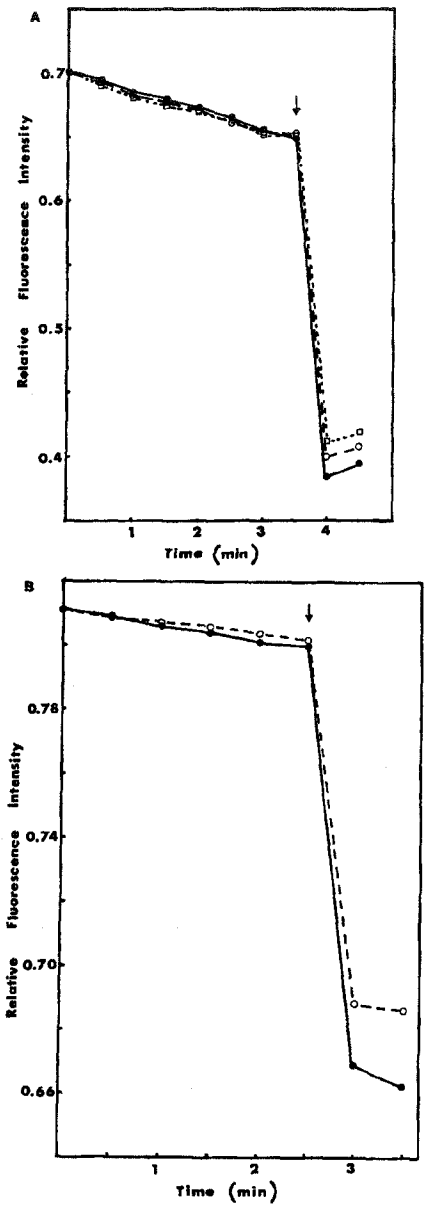

FIG. 5. **Measurement of passive proton flux in reconstituted vesicles or native membranes containing V_0.** *A*, peak gradient fractions containing V_0 (12 μg of protein) were obtained following detergent-solubilization of either native stripped vesicles (□) or membranes treated with KI and ATP (○) as described in Fig. 1. Reconstitution was then carried out either in the presence (□, ○) or absence (●) of protein as described under "Experimental Procedures." Reconstituted vesicles (25 μl containing 0.6 μg of protein) which had been prepared in buffer containing 150 mM KCl were then diluted 1:20 into buffer containing NaCl in place of KCl plus 2 μM ACMA. At t = 0, membrane potential driven proton flux was initiated by addition of 20 nM valinomycin and proton uptake was monitored by fluorescence quenching of ACMA (λ_{ex} = 410 nm, λ_{em} = 490 nm). At the *arrow*, the vesicles were made permeable to protons by the addition of 1.0 μM CCCP. *B*, stripped vesicles (1.5 mg of protein/ml) that had been treated with KI and ATP as described in Fig. 1 were loaded with 100 mM K_2SO_4 as described under "Experimental Procedures" and then treated for 1 h at 4 °C either in the absence (●) or presence (○) of 50 μM DCCD. Vesicles (25 μl containing 38 μg of protein) were then diluted 1:20 into buffer containing Na_2SO_4 in place of K_2SO_4 and, at t = 0, potential driven proton uptake was initiated by addition of 20 nM valinomycin and monitored by fluorescence quenching of ACMA as described above. At the *arrow*, the vesicles were permeabilized to protons by addition of 1.0 μM CCCP.

of the 100- and 38-kDa subunits to proteolytic cleavage.

Why there is an excess of V_0 domains in coated vesicles remains uncertain. It does not seem to be due to loss of the corresponding V_1 domains during dissociation of the clathrin coat or detergent solubilization and density gradient sedimentation of the (H^+)-ATPase, since Western blot analysis of fractions obtained throughout this procedure using the monoclonal antibody 3.2-F1 (specific for the peripheral A subunit (30)) does not indicate any significant loss of V_1 during these steps (data not shown). It is possible that some loss of V_1 domains occurs during isolation of the clathrin-coated vesicles from bovine brain. Alternatively, there may exist a pool of unassembled V_0 domains in the cell. Consistent with this idea is the observation that in MDBK cells we are able to detect a pool of unassembled V_1 domains.[2] The possible implications of these findings for regulation of vacuolar acidification are discussed below.

It has been demonstrated by a number of laboratories (35–37) that the F_0 domain of the F_1F_0 (H^+)-ATPases can act as a passive DCCD-inhibitable proton channel. Because of the similarity between the vacuolar and F_1F_0 classes of (H^+)-ATPase, both in overall structure (17–19) and in sequence homology (20–26), it was of interest to determine whether the V_0 domain, like F_0, could conduct protons. We investigated this question using both the isolated, reconstituted V_0 domain and native membrane vesicles from which the V_1 domains had been removed by treatment with KI and ATP (19). Proton movement was driven using a K^+/valinomycin-induced membrane potential and measured using uptake of the fluorescence dye ACMA. As can be seen in Fig. 5, in neither case did the V_0 domain exhibit DCCD-inhibitable proton translocation. We have shown previously that following dissociation of the peripheral subunits with KI and ATP, the V_0 domain remaining in the membrane is competent to assemble with V_1 to form a functional (H^+)-ATPase (27). Thus treatment with KI and ATP has not rendered the V_0 domain nonfunctional. These results suggest that, unlike F_0, the free V_0 domain is not an open proton channel.

This absence of proton conduction by V_0 is interesting in a number of respects. First, it has been reported previously that the 17-kDa c subunit of the coated vesicle (H^+)-ATPase, when extracted with toluene and reconstituted into phospholipid vesicles, was itself competent to form a DCCD-inhibitable proton channel (38). Although surprising in light of the lack of channel activity of the isolated homologous c subunit of F_0 (37), if correct this result would suggest that one or more of the remaining V_0 subunits is supressing the channel activity of the c subunit. A second question concerns why if the fully assembled F_0 domain conducts protons, the corresponding V_0 domain does not. One possible answer is that the cell employs assembly of V_1V_0 as a mechanism of controlling vacuolar acidification. According to this model, acidification would be activated in a particular compartment by attachment of V_1 to free V_0 domains preexisting in the corresponding membrane. It would be important under these circumstances that free V_0 not conduct protons since otherwise, in any membrane containing multiple V_0 domains, it would be impossible to establish any significant pH gradient until all of the available V_0 sites had been occupied. Whether free V_0 domains exist in the cell and what factors might control assembly of V_1 and V_0 *in vivo* remain important but unanswered questions.

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[2] M. Myers and M. Forgac, manuscript in preparation.

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